

SEPARATION AND ISOLATION OF (S)-EQUOL: THE BIOLOGICALLY RELEVANT  
METABOLITE OF THE ISOFLAVONE DAIDZEIN

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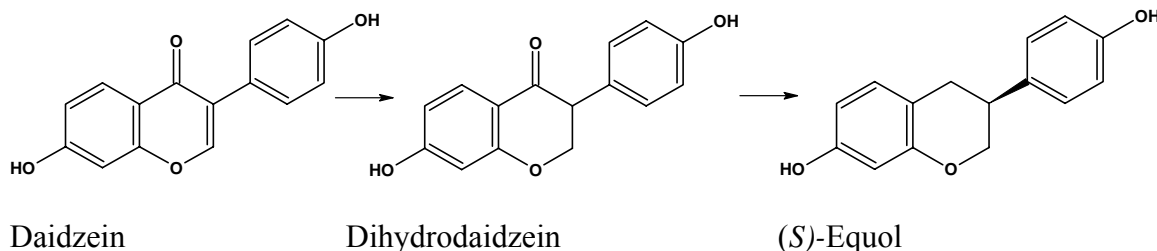
## ABSTRACT

Isoflavones, a class of phytochemicals abundant in soy, have recently been investigated for their biological activity in humans. One of the primary soy isoflavones, daidzein, is transformed into *S*(-)-equol, a metabolite produced by microflora in the human intestine. Synthesized equol is currently available as a racemic mixture, while only the biologically active *S*(-)-equol is created by bacteria in humans. Because of the interest in this compound, a need exists for isolating and identifying the *S*(-) isomer for further analytical and biological studies. The objective of this study is to create a simple, accurate, and cost effective High Performance Liquid Chromatography (HPLC) method for separating the two enantiomers of equol using easily obtainable compounds. (+/-)-Equol was purchased and reacted with methoxytrifluorophenyl acyl chloride to produce diastereomers. The diastereomers were analyzed for purity by HPLC. Successful derivatization of the equol-MTPA esters was confirmed by HPLC-Mass Spectrometry (MS). The two diastereomers were separated via HPLC on a Luna Silica column (4.6 mm x 150 mm, 3  $\mu$ m packing), 1 mL/min flow at 25°C, gradient = 1/99 MtBE:hexane to 4.3/96.7 MtBE:hexane over 49.5 min, and separation was confirmed with HPLC-MS. Next, fractions will be collected of each diastereomer, the MTPA will be removed, and Circular Dichroism Spectra analysis will be performed on both collections to ensure separation has been achieved.

## BACKGROUND

The compound known as equol was first discovered in pregnant mare's urine in 1932 by two British scientists, Marrian and Haslewood, who were attempting to isolate and crystallize estradiol. Equol was present as an impurity, which they further investigated to determine its chemical structure. Little additional research was done on the molecule until 1946, when equol was associated with sheep infertility in Southwestern Australia (Bennets 1946). It was found that the sheep were consuming red clover containing an abundance of formononetin, which is converted into equol in the gut. The equol exhibited estrogen-like effects on the sheep.

Since its discovery, we have learned much more about equol and its origins. Phytoestrogens, or “plant-estrogens” are molecules which are naturally found in plants and mimic estrogen in mammals after heavy consumption and absorption into the body. Equol is a derivative of daidzein, one such phytoestrogen. Kudzu, red clover, and soy products are the most predominant sources of equol precursors, which fall into a class of structures called isoflavones (Benlihabib 2004, Kazi 1984). After ingestion, microflora in the intestine convert daidzein to equol in a two step process (Rowland 2000) shown in Figure 1. The bacteria involved in this metabolic process are still being investigated, although to date *Eggerthella sp.* strain Julong 732 and *Clostridium sp.* strain HGH6 have been shown to perform separate steps of the reaction *in vitro* (Wang 2005). It is estimated that 30%-50% of the human population harbor bacteria which perform this reaction (Setchell 2002).



**Figure 1.** The conversion of Daidzein to (S)-Equol occurs via microflora in the large intestine. The mechanism and bacteria involved are still being investigated.

As previously mentioned, phytoestrogens mimic estrogen in the mammalian body. The drop in estrogen during menopause is associated with a wide range of diseases, including an increased risk for developing osteoporosis, cardiovascular disease, and cancer (Cheung 2004). Soyflour has been shown to reduce bone resorption in postmenopausal women (Murkies 1995). Potter and Colleagues have also found that 40g of isoflavone rich soy protein consumed by postmenopausal women every day for six months increased the bone mineral density of the lumbar spine, whereas bone mineral density decreased in the control group consuming 40g/day of casein-based milk protein (1998). In the same study by Potter et al (1998), the isoflavone rich protein increased HDL levels while decreasing total cholesterol. Besides the positive contributions to bone and heart health, isoflavones have additional health benefits. In fact, increased levels of isoflavones have been associated with a decreased risk of developing hormone dependent cancers (Adlercreutz 1992).

### **Equol**

While research continues on isoflavones, it has been suggested that the metabolite equol may in fact be a more potent agonist or antagonist *in vivo* than its precursors or other isoflavones. Equol, and the highly abundant isoflavone genistein, have been found to bind with comparable affinity to ER $\alpha$ . However, equol induces more transcription at this receptor (Morito 2001). In one randomized crossover study, premenopausal women were given soy protein, soy isoflavone supplements, and placebo for three menstrual cycles. Plasma hormone profiles were determined, and women who produced equol had hormone profiles associated with a lower risk for breast cancer (Duncan 2000). In a separate study, performed by Uesugi and colleagues, 58 menopausal Japanese women were given 40mg/d of isoflavones for four weeks. Equol producers in this study showed a significant decrease in urinary deoxypyridinoline, a marker of bone resorption

(2004). While these studies provide evidence to suggest that equol may induce positive biological outcomes *in vivo*, much work needs to be done to determine exactly what these effects are for each optical isomer and how they are achieved.

### **Chirality**

Equol is classified as a chiral molecule. A chiral molecule is defined as a molecule which is not superimposable on its mirror image (Carey 2003). In other words, the two mirror image forms have a different spatial or three dimensional configuration. A pair of non-superimposable mirror image molecules are called enantiomers.

Chiral molecules, or “mirror image” molecules, rotate polarized light in different directions. In contrast, racemic mixtures do not rotate polarized light (Carey 2003).

Chiral molecules are ubiquitous throughout the plant and animal kingdom. In fact, except for glycine, the human body produces only one of the two enantiomers possible for each amino acid (Voet 2002).

This stereo selectivity in synthesis also has implications for receptor recognition. It is quite common for one enantiomer to produce strong effects *in vivo*, while its counterpart has no effect or adverse effects. For example, the drug Thalidomide was commonly prescribed in the 1960's for the alleviation of nausea in pregnant women. Thalidomide is a chiral molecule, and when synthesized produces a racemic mixture of *R* and *S* forms. The active enantiomer successfully reduced morning sickness symptoms; however, the “inactive” enantiomer was later discovered to cause birth defects in children born to women who took the drug (Voet 2002). This example illustrates the importance of isolating and separately investigating each enantiomer of an unknown product.

## **Chiral Separation through Derivatization**

Chiral derivatization has been a widely used method for creating diastereomers which can be separated via gas chromatography or HPLC (Lindner 1988). Briefly, a racemic mixture of the compound of interest is reacted with an optically active reagent to create covalent bonds. The product of this reaction is two diastereomers. Due to differing physiochemical properties, each diastereomer should interact differently with an achiral HPLC column and elute at separate times (Ahnoff 1989).

This method requires the presence of functional group on the enantiomeric compound to react with the optical separating agent, which should be available in high purity, react quantitatively, does not racemize during storage, and yield derivatives which do not spontaneously racemize (Lindner 1988). The best resolution between diastereomers is achieved when the chiral centers of the resulting diastereomer are in close proximity. Close chiral centers maximize the difference in stereo chemical structure between the two diastereomers allowing for better separation (Lindner, 1988). In addition, bulky, rigid derivatizing reagents help to hold the resulting molecules in a stiff conformation, enhancing separation (Lindner 1988).

While the derivatization technique has become less popular since the advent of more sophisticated methods utilizing chiral columns or selective enzymatic reactions, it remains an inexpensive and effective method of separating enantiomers by HPLC.

## **Reverse Phase HPLC**

In reverse phase chromatography (RPC), the sample of interest is partitioned between a non-polar stationary phase and a polar solvent system. The relative affinity of the sample for the stationary and mobile phases determines the retention time. More hydrophobic compounds are retained on the column longer than hydrophilic compounds, which have a greater affinity for the

mobile phase. Compound polarity, mobile phase characteristics, column characteristics, and temperature all affect the retention time of the compound (Snyder 1997).

Column characteristics which can alter the affinity of the sample for the column include the type of bonded phase (typically C<sub>8</sub>, C<sub>18</sub>, or C<sub>30</sub>), the concentration of the bonded phase, and the surface area of the column. An increase in column temperature slightly reduces retention time in most instances and sharpens peaks (Snyder 1997).

RPC is the preferred choice for separation of most samples, due to its rugged and robust nature. RPC offers methods which provide adequate separation, stable columns, fast, and reproducible methods, as well as easier detection due to the lower UV cutoff for most solvents (Snyder 1997).

### **Normal Phase HPLC**

Normal phase chromatography (NPC) utilizes a polar stationary phase and a non-polar organic mobile phase to separate different compounds. The stationary phase typically consists of silica, but cyano, diol, and amino columns are also common. In contrast, the mobile phase usually contains one non-polar solvent, like hexane, and one weakly polar solvent like isopropanol or ethyl acetate (Snyder 1997).

The separation process is believed to occur via adsorption. Initially, the polar component of the mobile phase is adsorbed as a single layer covering the highly polar surface of the stationary phase. When the sample of interest is injected onto the column, the sample components displace this adsorbed solvent. As more solvent flows through the system, the polar solvent slowly replaces the sample at the adsorption sites, attacking the weakest associations first. Consequently, the elution order of the sample components usually follows from highly nonpolar to highly polar (Snyder 1997).

Through manipulation of the mobile phase components, the elution time and order of the sample compounds can be altered. In normal phase chromatography, polar compounds and solvents tend to attach strongly, or “localize” onto an adsorbent site. In contrast, nonpolar solvents and samples do not exhibit localization on the column. Knowledge of solvent and sample leads to a better choice of mobile phase solvents. For example, if the compound of interest is highly localized on the stationary phase, then a localized solvent will compete with the compound and selectively displace it faster than a nonlocalized solvent (Snyder 1997).

With the diverse array of organic solvents and column packings available for normal phase chromatography, it is often preferred over reverse phase chromatography for greater selectivity in separation. The superior selectivity of NPC in comparison to RPC also carries over into the separation of stereoisomers (Snyder 1997).

NPC does present a number of challenges which must be addressed for successful utilization of this method. To begin with, the silica stationary phase used most often in NPC is highly sensitive to any water which may have accumulated in the mobile phase from the air. Water adsorbs strongly to the column and can severely alter sample retention times. In addition, the amount of time necessary for re-equilibration of a column is much longer than the time necessary for equilibration in RPC, especially when a polar localizing solvent is being replaced with a highly nonpolar mobile phase. Finally, solvent demixing may be observed when a gradient is employed in NPC. When the polar component of the mobile phase is slowly increased, it is adsorbed onto the surface of the stationary phase until the stationary phase is completely saturated. At the saturation point, the percentage of polar solvent flowing through the entire column increases suddenly. This rapid change may elute some sample components with short retention times and produce poor separation (Snyder 1997).



## JUSTIFICATION

When Marrian and Harrison initially discovered equol, they measured the optical rotation of newly discovered equol determining early on that equol is a chiral molecule. Later work has shown that only the *S* enantiomer of equol is made in the body (Setchell 2002), but this information has not been applied to most of the *in vitro* research performed. Often, a synthesized racemic mixture of equol is used in these studies, containing a 1:1 ratio of the *R* and *S* forms of a compound. Consequently, the results from these studies may not accurately reflect the positive and negative effects of the molecule which would be found *in vivo*.

While a stereo-selective chemical synthesis method of *S*-equol has yet to be developed, two other methods have been published to date which produce *S*-equol. Muthyala and colleagues developed a high performance liquid chromatography (HPLC) method for the separation of racemic equol standard (2005). An analytical  $\beta$ -cyclodextrin column (chiral) with an aqueous mobile phase were used to separate the enantiomers; HPLC-MS (with an electrospray ionization interface) and Circular Dichroism Spectra were used to confirm the successful separation of the *R* and *S* forms of equol. Unfortunately, both analytical and preparatory chiral stationary phase HPLC columns are very expensive. The costs involved render this method impractical for the majority of researchers investigating this compound.

In contrast, the second method involves the *in vitro* cultivation of an anaerobic gram-negative bacteria *Eggerthella sp.* strain Julong 732. This bacteria, obtained from human fecal samples, converts dihydrodaidzein into *S*-equol (Wang 2005). Again, this method presents some challenges. The amount of bacteria necessary to generate a material amount of *S*-equol for *in vitro* testing is quite large. In addition, a number of impurities would be present in the extracted product.

We propose a simple, accurate, and cost effective method of separating the two enantiomers of equol from the racemic, artificially synthesized standard. Derivatization of the standard with another chiral molecule creates diastereomers, whose stereo structures should separate on a traditional HPLC column. Following separation, removal of the derivatizing reagent will liberate the individual enantiomers of equol.

## **MATERIALS + METHODS**

### **Materials**

Racemic (+/-)-Equol standard was purchased from LC Laboratories (Woburn, MA). (*S*)-(-)-Camphanic Chloride, (*S*)-(+)-Camphorsulfonyl Chloride, (*S*)-(+)-Methoxytrifluorophenylacetyl Chloride, anhydrous pyridine, and 4-dimethylaminopyridine were purchased from Sigma-Aldrich (St. Louis, MO). Triethylamine and all solvents (HPLC grade) were purchased from Fisher Scientific (Hampton, NH). Ultraviolet Spectrometry was performed on a Shimadzu UV-2401 PC Spectrophotometer.

### **Experimental Derivatization**

To a solution of 0.04 mmol of racemic equol standard and 0.22 mmol of pyridine dissolved in 200  $\mu$ L of dichloromethane was added dropwise a solution of 0.20 mmol of acyl chloride in 200  $\mu$ L of dichloromethane, capped and shaken for 16 hrs. Needlelike crystals were observed upon combining reagents. The reaction mixture was dried under nitrogen gas, and then the remaining material was resolubilized in 0.5mL of dichloromethane. The this solution was extracted 3 x 0.5 mL saturated sodium carbonate solution, 1 x 0.5mL brine, dried over sodium sulfate and filtered. Solvent was removed from the filtrate under nitrogen gas.

## **HPLC Methods**

All product samples were tested using a Waters 2695 System with 996 photodiode-array (PDA).

Solvent A: acetonitrile, solvent B: 1% acetic acid, solvent C: methyl-tert-butyl-ether (MTBE)

solvent D: hexane. HPLC Method 1: Waters Novapak C18 (3.9mm x 150mm, 4 $\mu$ m packing)

column, 1 mL/min flow at 25°C, gradient = 5/95 A:B to 95/5 A:B over 15 min, 95/5 A:B for 5

min. HPLC Method 2: Phenomenex Luna Silica column (4.6 mm x 150 mm, 3  $\mu$ m packing), 1

mL/min flow at 25°C, gradient = 1/99 C:D to 4.3/96.7 C:D over 49.5 min, up to 50/50 C:D over

1 minute, held 50/50 C:D over 9.5 min

## **HPLC-MS Methods**

The equol-dicamphanic acid esters and equol-dicamphorsulfonyl esters were analyzed on a

Waters 2695 HPLC system, 996 PDA UV/visible absorbance detector interfaced via a flow

splitter (0.1 mL/min into MS) to a Quattro Ultima quadrupole mass spectrometer (Micromass

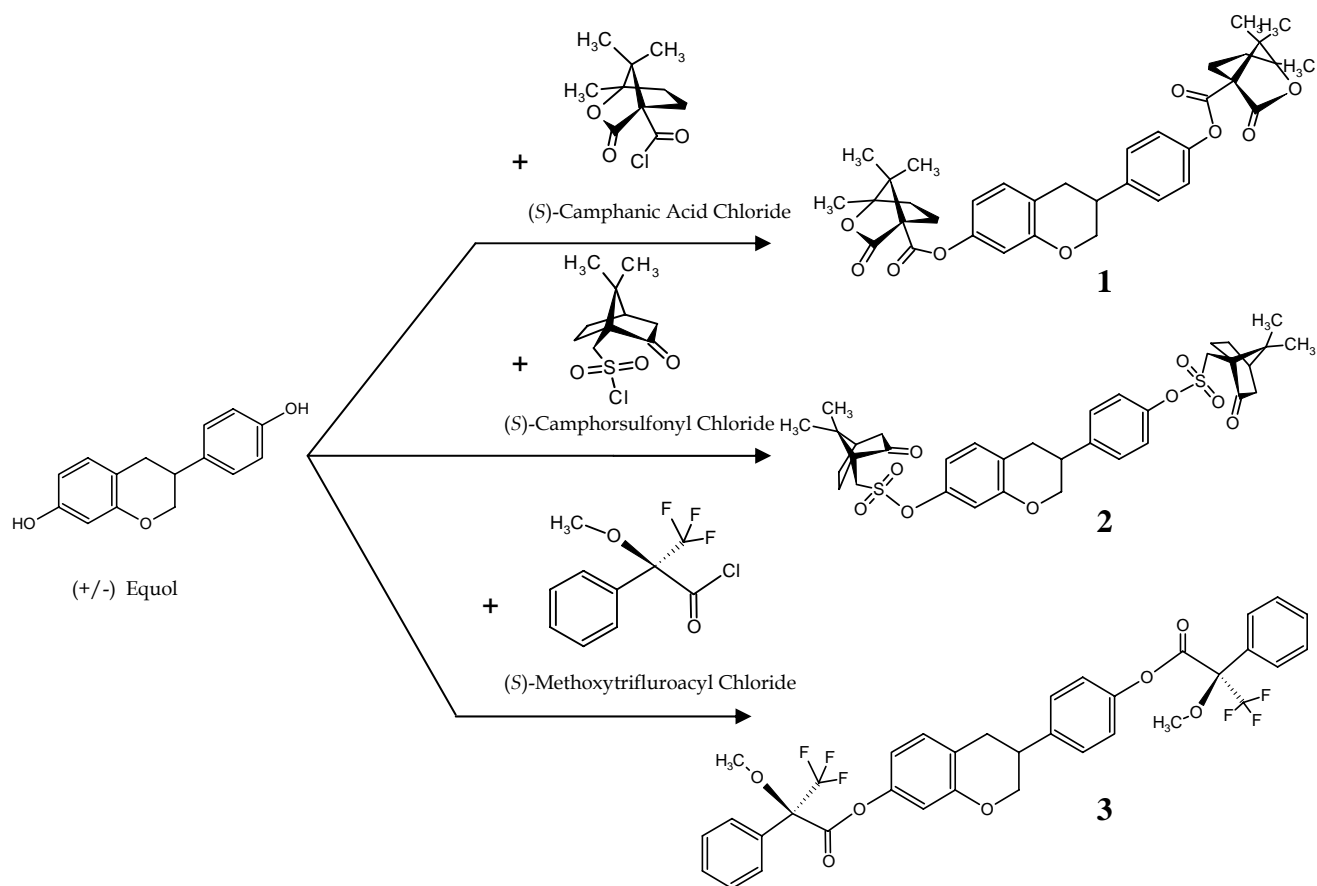
Limited, Manchester,UK). Equol-MTPA esters were analyzed using HPLC method 2 on an

HP1100 with a diode-array detector (DAD) system, coupled to a QToF I mass spectrometer

(Micromass Limited, Manchester,UK) via a flow splitter deliver eluent at 0.1 mL/min. The MS

interface was atmospheric pressure chemical ionization (APCI). Instrument control and data

analysis were accomplished with Masslynx V3.5 software.



**Scheme 1.**

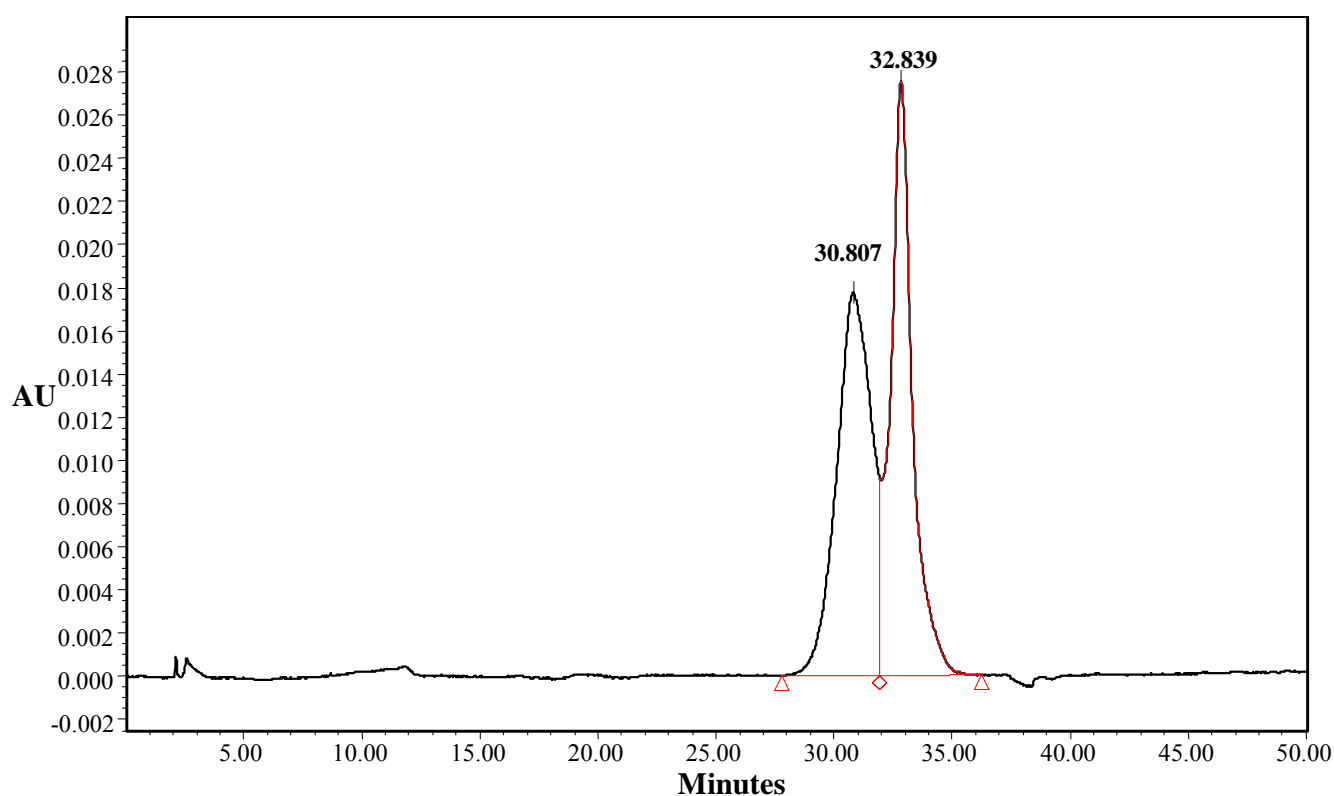
## RESULTS

**Equol-dicamphanic acid ester (1)** A single compound was observed via HPLC based on appearance of product at 254 nm; retention time, HPLC method 1: 14.11 min.  $\lambda_{\text{max}}$  (taken from HPLC) = 277.5 nm. Mass spectra showed a  $m/z = 601$   $[M-H]^-$  (spectra not shown) as the parent ion confirming the formation of compound **1**. Work to separate the two diastereomers was first attempted with RPC. C18, C8, and CN columns, and various solvent combinations, including acetonitrile/1% acetic acid, methanol/acetonitrile/1% acetic acid, methanol/1% acetic acid, tetrahydrofuran/1% acetic acid were tested. No separation was observed. Next, NPC was tested using a silica column and mobile phase combinations including hexane/ethyl acetate, hexane/ethyl acetate/dichloromethane, dichloromethane/methanol, dichloromethane/propanol, and dichloromethane/ethyl acetate. Again, no separation was achieved.

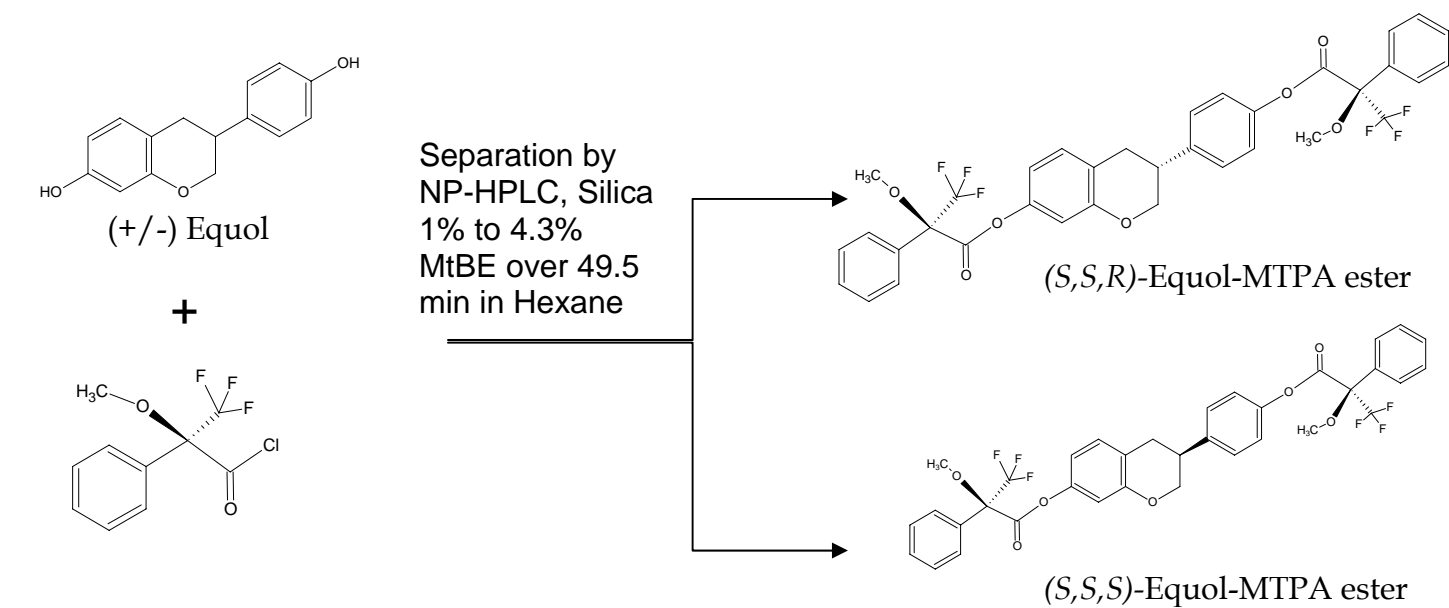
**Equol-dicamphorsulfonyl ester (2)**. A single compound was observed via HPLC based on the appearance of product at 254 nm; retention time, HPLC method 1: 14.90 min.  $\lambda_{\text{max}}$  (taken from HPLC) = 277.5 nm. Mass spectra showed a  $m/z = 672$   $[M-H]^+$  (spectra not shown) as the parent ion, confirming the formation of compound **2**. The derivatives were briefly tested using a C18 column and acetonitrile/1% acetic acid. The retention times for the equol-dicamphorsulfonyl esters were almost identical to the retention times of the dicamphenate derivatives. No separation was observed.

**Equol-MTPA ester (3)**. A major component of the reaction was observed via HPLC with a retention time, HPLC method 1: 17.55 min.  $\lambda_{\text{max}}$  (taken from HPLC) = 277.5 nm. Mass spectrograms showed the major component to have a  $m/z = 675$   $[M-H]^+$  (spectra not shown). RPC with a C18 and CN column was tested with mobile phase solvent combinations including acetonitrile/1% acetic acid, methanol/1% acetic acid, and tetrahydrofuran/1% acetic acid. No

diastereomeric separation was observed. NPC was tested using a silica column and ethyl acetate, ethyl acetate/hexane, and isopropanol hexane, but no separation was observed. HPLC method 2 was found to separate the single product peak into two peaks eluting between 29 min and 35 min with a ratio of 1.05:1.00 peak area, as shown in Figure 2. A schematic of the separation is shown in Scheme 2. The two peaks produced in Figure 2 have identical UV spectra, as shown in Figure 3.

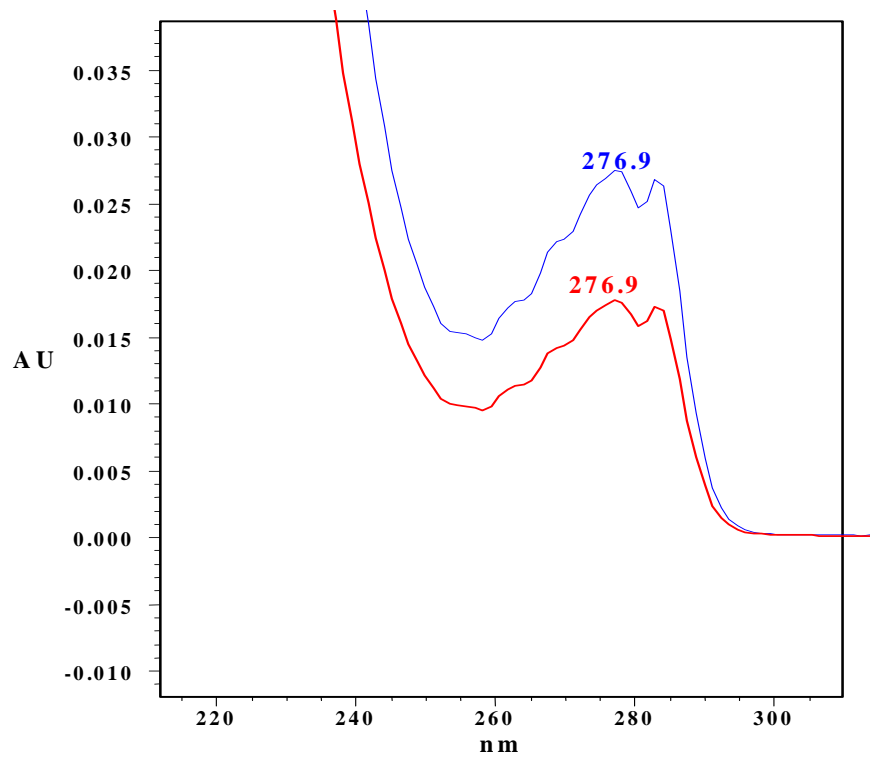


**Figure 2.** The separation of the two equol-MTPA ester diastereomers (compound **3**) using HPLC method 2.



(S)-Methoxytrifluoroacetyl Chloride

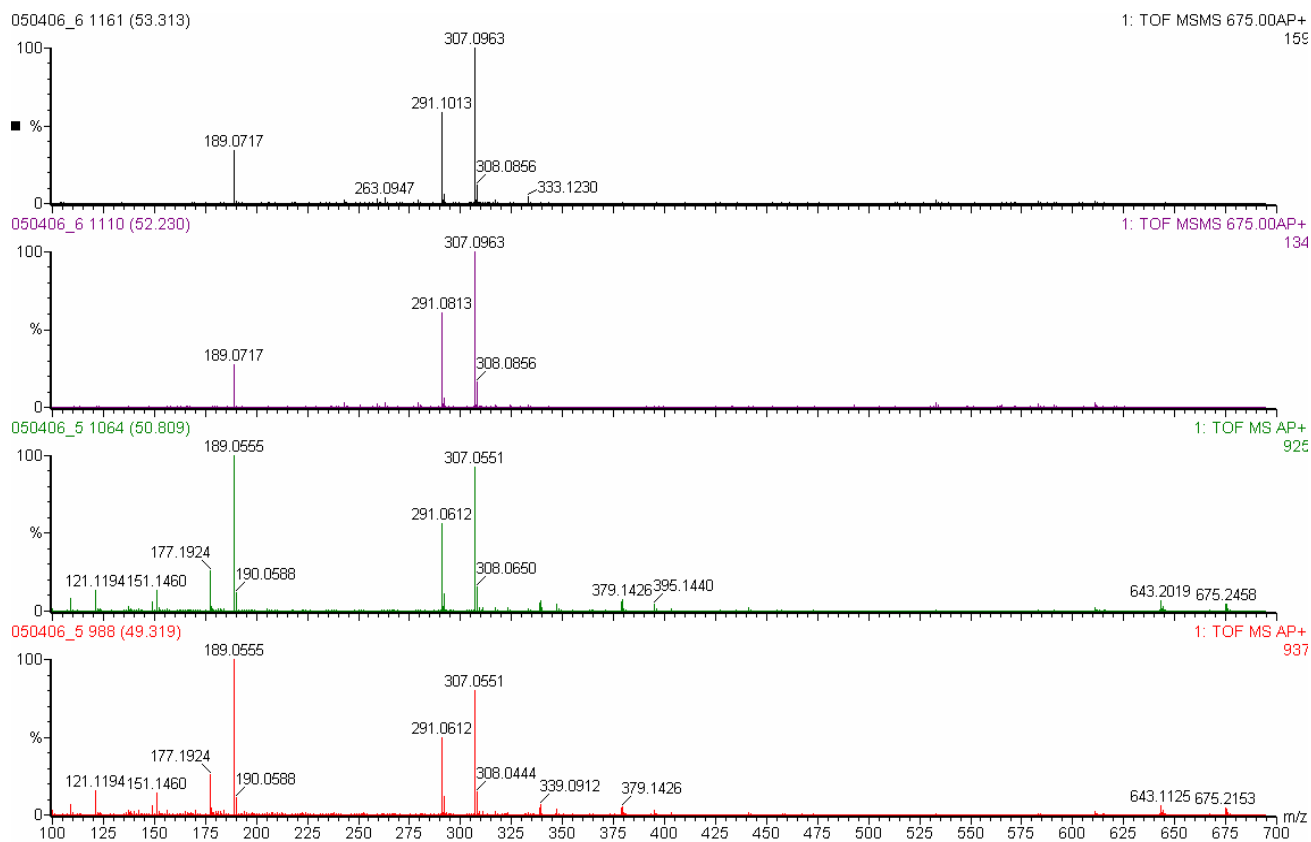
**Scheme 2.**



**Figure 3.** Overlapping UV spectra taken of the two peaks depicted in Figure 2.

MS analysis revealed the successful separation of two compounds with identical m/z ratios.

HPLC-MS/MS testing showed identical fragmentation patterns between both compounds (Figure 4).



**Figure 4.** Mass Spectra obtained from the separation of compound 3. Spectra 1 and 2 were performed in HPLC-MS/MS mode, and illustrate identical fragmentation patterns. Spectra 3 and 4 were taken from HPLC-MS and indicate the parent ion at 675 m/z.



## DISCUSSION

Camphanic acid chloride was chosen as the first derivatizing agent due to its use in the successful separation of astaxanthin isomers (Vecchi and Müller, 1979). The reaction was fast and simple to perform. After compound **1** was proven with HPLC-MS to be successfully synthesized, work began to separate the two diastereomers on HPLC. Both RPC and NPC were tested. The solvent gradient, injection solvent, flow rate, temperature, and injection volume were manipulated to optimize separation. Each mobile phase method was tested until the product peak broadened to a point at which diastereomeric separation was clearly unobtainable. However, no separation was observed with the methods employed.

Some of the difficulty encountered in separating compound **1** can be attributed to the poor solubility of the product in most solvents. Acetonitrile, dichloromethane, and tetrahydrofuran were the only solvents in which the camphanic acid diastereomers dissolved in a material amount. Consequently, the mobile phase solvents available to test on HPLC were limited.

Next, we tried using camphorsulfonyl chloride as a derivatizing reagent. We hypothesized that the sulfonyl group might interact uniquely with the stationary phase. Compound **2** was synthesized in analogous method to compound **1**, and brief testing revealed that the behavior of compounds **1** and **2** were very similar on HPLC. Again, no separation was observed.

MTPA was chosen as the third derivatizing reagent due to the bulky functional groups and the widely reported success of diastereomeric separation with this reagent. RPC testing was

initially tried, but no separation occurred. NPC was attempted next, and after testing multiple solvent systems, a combination of MtBE and hexane (HPLC method 2) was found to elute two peaks at 30.807 min and 32.839 min. Both peaks have identical UV spectra, and the ratio of the peak areas (1.05:1) is indicative of diastereomeric separation. Additional MS analysis revealed the successful separation of two compounds with identical m/z ratios. HPLC-MS/MS testing showed identical fragmentation patterns between both compounds. This data supports the conclusion that we have in fact separated the two MTPA-equol diastereomers.

## CONCLUSIONS

We have successfully synthesized three classes of diastereomers through the reaction of (+/-) equol with three acyl chlorides: (*S*)-camphanic acid chloride, (*S*)-camphorsulfonyl chloride, and (*S*)-methoxytrifluorophenylacyl chloride. All derivatives were tested via HPLC-MS to ensure successful synthesis. HPLC methods were employed to separate the synthesized diastereomers. Successful separation was achieved with the equol-(*S*)-methoxytrifluorophenyl esters using a silica column and a gradient of MtBE/hexane. HPLC-MS confirmed that both peaks produce the same UV spectra, the same parent ion, and the same fragmentation patterns. Once each optical isomer is obtained, their relative biological activity can be measured and compared both *in vitro* assays and *in vivo* assays.

## FURTHER WORK

In order to further confirm that separation of the diastereomers has occurred, fractions of each diastereomer must be collected and pooled. The MTPA will be cleaved with a base, leaving the separated *R* and *S* forms of equol. The enantiomeric purity will be evaluated by Circular Dichroism spectra.

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